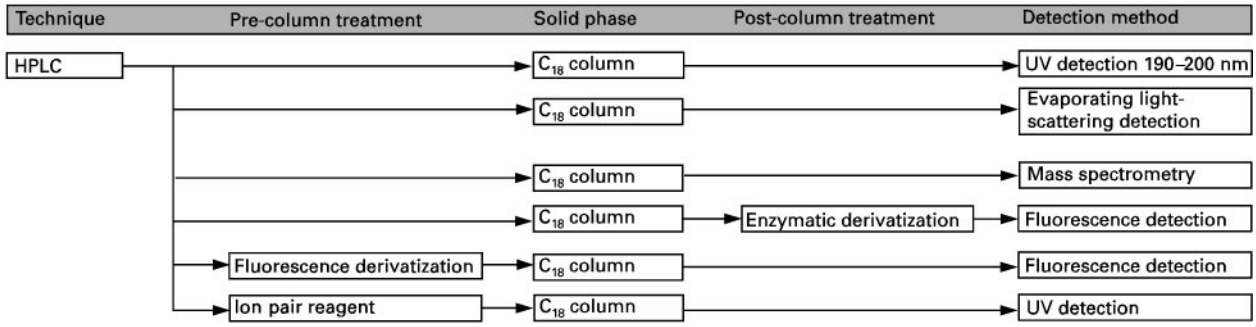

Figure 3 Separation methods for bile acids by liquid chromatography.

higher quantities. For these samples the standard purification procedure using C_{18} solid-phase extraction is suitable.

Due to the low concentration range of the bile acid pattern in serum and urine, the methods of choice for quantitative and reproducible determination are pre-column fluorescence derivatization and HPLC-MS coupling.

Conclusion

With regard to the liquid chromatographic methods described for bile acid analysis, especially with regard to their determination in biological matrices, several approaches for qualitative and quantitative determination have been described. A critical evaluation of the advantages and disadvantages of these methods results in the conclusion that there are specific limitations for every application (**Figure 3**). Since cumbersome derivatization steps as well as laborious and unsuitable sample pretreatment play a key role in the value of the analytical results, technologies are needed where direct analysis of the bile acid pattern, especially in serum, is feasible. Only HPLC-MS coupling presently allows such analysis, but costly equipment and highly skilled personnel requirements limit this method to highly specific problems and makes it unsuitable for routine work.

Presently, HPLC of bile acids is state-of-the-art and only incremental improvements can be expected from new column materials and microcolumns ($\varnothing$ 1–2 mm). The future will be in the area of low cost, high throughput HPLC-MS devices for routine use.

See also: **II/Chromatography:** Liquid: Derivatization. Detectors: Evaporative Light Scattering; Detectors: Fluorescence Detection; Detectors: Mass Spectrometry; Detectors: Ultraviolet and Visible Detection. **III/Bile Acids:** Gas Chromatography.

Further Reading

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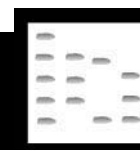
BILE COMPOUNDS: THIN-LAYER (PLANAR) CHROMATOGRAPHY



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Introduction

Gall bladder bile is, like other physiological fluids, of very complex nature. It is produced by liver

hepatocytes of healthy human beings in a volume of 500–1000 mL per day. The major function of the bile fluid is to support digestion and resorption of lipids and lipophilic substances during intestinal passage. In this respect, bile acids and their different conjugates are the most important components. Bile acids are steroids of amphophilic nature and, owing to their chemical structure, tend to form micelles and serve as physiological detergents. It is this feature that makes bile acids so important in their role in emulsifying fatty food components. In addition, bile acids serve as enzyme cofactors and support fat digestion by activating lipases and other factors of lipid metabolism. Humans, together with some other animals, possess a gall bladder in which bile fluid is stored. The human gall bladder has a capacity of 15–20 mL of concentrated bile. When bile is needed for digestion excretion into the gut is triggered by cholecystokinin, a gastrointestinal hormone which causes ejection of gall bladder contents. In the gall bladder bile is concentrated five- to ten-fold, leading to increased concentrations of all major bile components. Total bile production as well as bile composition are highly dependent on amount and composition of food. For example, according to differences in eating, the pH values of human bile range from 6.5 to 8.5. Other animals, such as rats, secrete bile fluid directly into the intestine.

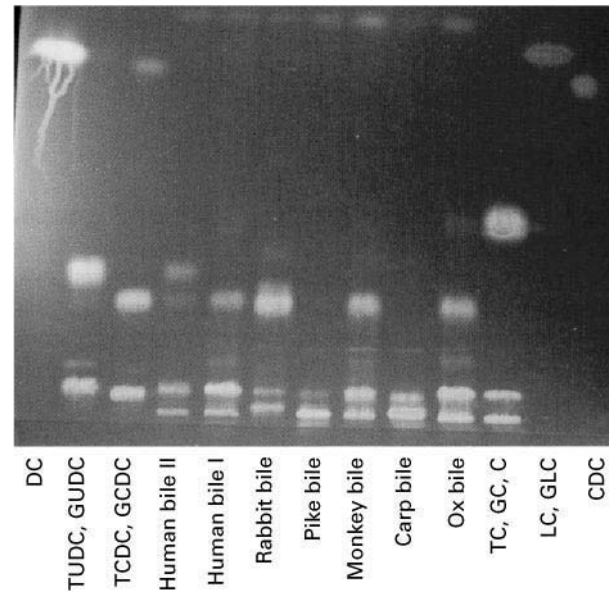

Figure 1 Thin-layer chromatogram of gall bladder bile from several animals, and of human T-drainage bile; comparison with selected standard bile acids. C, cholic acid; DC, deoxycholic acid; CDC, Chenodeoxycholic acid; GC, glycocholic acid; GCDC, glycochenodeoxycholic acid; TC, taurocholic acid; TCDC, taurochenodeoxycholic acid; TUDC, tauroursodeoxycholic acid; GUDU, glycoursodeoxycholic acid; LC, lithocholic acid; and GLC, glycolithocholic acid. (From Müller *et al*. 1992.)

The most important bile components are the bile acids and their different amino acid conjugates. Bile acids are synthesized in the liver from cholesterol and therefore play an additional key role in cholesterol homeostasis. Products of this process are the so-called primary bile acids cholic acid and chenodeoxycholic acid. In the bile fluid mainly glycine and taurine conjugates of these primary bile acids as well as those of deoxycholic acid are present. However, after secretion into the gut primary bile acids are modified by the intestinal microflora. Primary bile acids and all their derivatives produced during intestinal passage undergo enterohepatic circulation, by which bile acids are reabsorbed nearly completely and are transported back to the liver. Only a very small part of the bile acid pool, 2–8%, is excreted with the faeces. Nevertheless, the bile of healthy individuals contains more primary than secondary bile acids. As changes in total bile acid amount as well as in the bile acid pattern are of importance for diagnostic purposes, simple and fast methods for the analysis of these biological compounds are required (**Figure 1**).

Many drugs and drug metabolites are secreted into the bile, and this, in addition, makes bile analysis important for the understanding and controlling of pharmacokinetic mechanisms during drug development. Several methods using high performance liquid chromatography (HPLC) and thin-layer chromatography (TLC), have been described for the analysis of pharmacologically interesting substances and their metabolites in bile, but as the analytical conditions are always dependent on the chemical nature of the drug and its concentration, they will not be considered here. Another bile component, cholesterol, has become increasingly important during recent decades. The role of increased serum cholesterol concentrations in diseases such as atherosclerosis, leading to stroke and cardiac infarction, for example, is the subject of debate, but when added to other risk factors its involvement is undisputed. Cholesterol concentrations in gall bladder bile range from 1 to 15 $g\,L^{-1}$ in humans. Increased bile cholesterol concentrations in most cases cause the formation of gallstones through cholesterol crystallization. The development of drugs influencing either serum or bile cholesterol concentrations is one of the most important areas in pharmaceutical research. Therefore, also in this field fast and simple methods for the detection and quantification of cholesterol in bile are necessary (**Figure 2**).

Bile fluid also contains high concentrations of phospholipids, especially lecithin, in amounts of 2–4 $g\,L^{-1}$. However, whether increases or decreases in phospholipid concentration are significant for

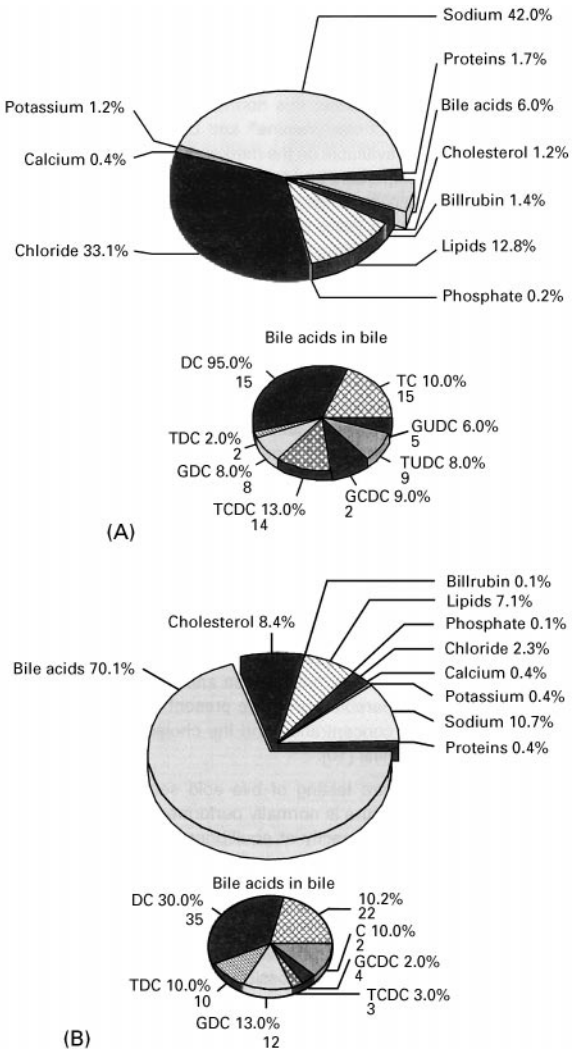

Figure 2 Composition of (A) human T-drainage bile (water content 95.5%) and (B) bovine gall bladder bile (water content 81.5%). (From Müller *et al.* 1992.)

differential diagnosis of certain enterohepatic diseases is not known. Nevertheless, the analysis of phospholipid pools and pattern in bile can be of value for metabolism and kinetic studies in drug development. Several TLC-based methods for separation and detection are known. Other bile components, such as bile pigments, proteins (e.g. alkaline phosphatase) and electrolytes, have to be analysed by other analytical methods (e.g. enzyme assays or spectrometric methods). In general, biliary protein concentration is negligible in healthy individuals (**Figure 3**).

Thin-Layer Chromatography

Bile Acid Separation

In contrast to other analytical technologies such as HPLC and gas chromatography, which are also commonly used for bile acid analysis, TLC is a very simple and nearly universal method. No highly sophisticated and expensive instrumentation is needed for reliable semiquantitative analysis. If precise quantification is essential, a TLC scanner equipped with a computer software system for fast and easy data registration and evaluation is recommended. Whereas HPLC analysis is limited by the fact that only one sample can be analysed at a time, TLC allows multiparallel analysis of probes in combination with an almost unlimited number of detection and visualization methods.

Several solvent systems for the separation of bile acids in biological fluids are described. With respect to bile, bile acid concentrations are in the millimolar range and therefore no detection problems occur, which makes TLC well suited for bile and bile acid analysis. In addition, time-consuming sample pretreatment procedures can be generally avoided for standard analysis of bile acids. However, dilution of the bile samples with phosphate-buffered saline increases the sensitivity of the selected detection (**Figure 4**).

For bile acid pattern analysis a satisfactory separation of the main components, the glycine- and taurine-conjugated bile acids, is necessary. In general, silica gel TLC plates are used for routine analysis since high quality materials are commercially available in several sizes and from several suppliers and, in contrast to reversed-phase plates for example, at a relatively low price. Our experience in this field leads us to suggest the use of silica gel pre-coated on glass plates with a concentration zone to improve separation, bile acid band shape and overall resolution. Solvent systems suitable for the separation of bile acids on silica gel are shown in **Table 1**. Many methods for bile acid separation have been described in the literature, and only a selection are shown here.

System 2 leads to the most satisfactory results in resolution and band shape. With this system the glycine and taurine conjugates of deoxycholic acid and of chenodeoxycholic acid can be separated with good resolution. The TLC plates have to be developed six times in the same solvent mixture for bile acid separation and this requires more time compared with the other solvent systems described, but if quantification by TLC scanning is followed system 2 is the method of choice. Solvent system 3 provides a simple and fast method for the separation of conjugated and unconjugated bile acids with sufficient overall resolution to allow quantification. The advantage of this system is the simultaneous separation of cholesterol, which migrates faster than the bile acids. Chamber saturation before analysis increases resolution and improves band shape. With system 4, a solvent

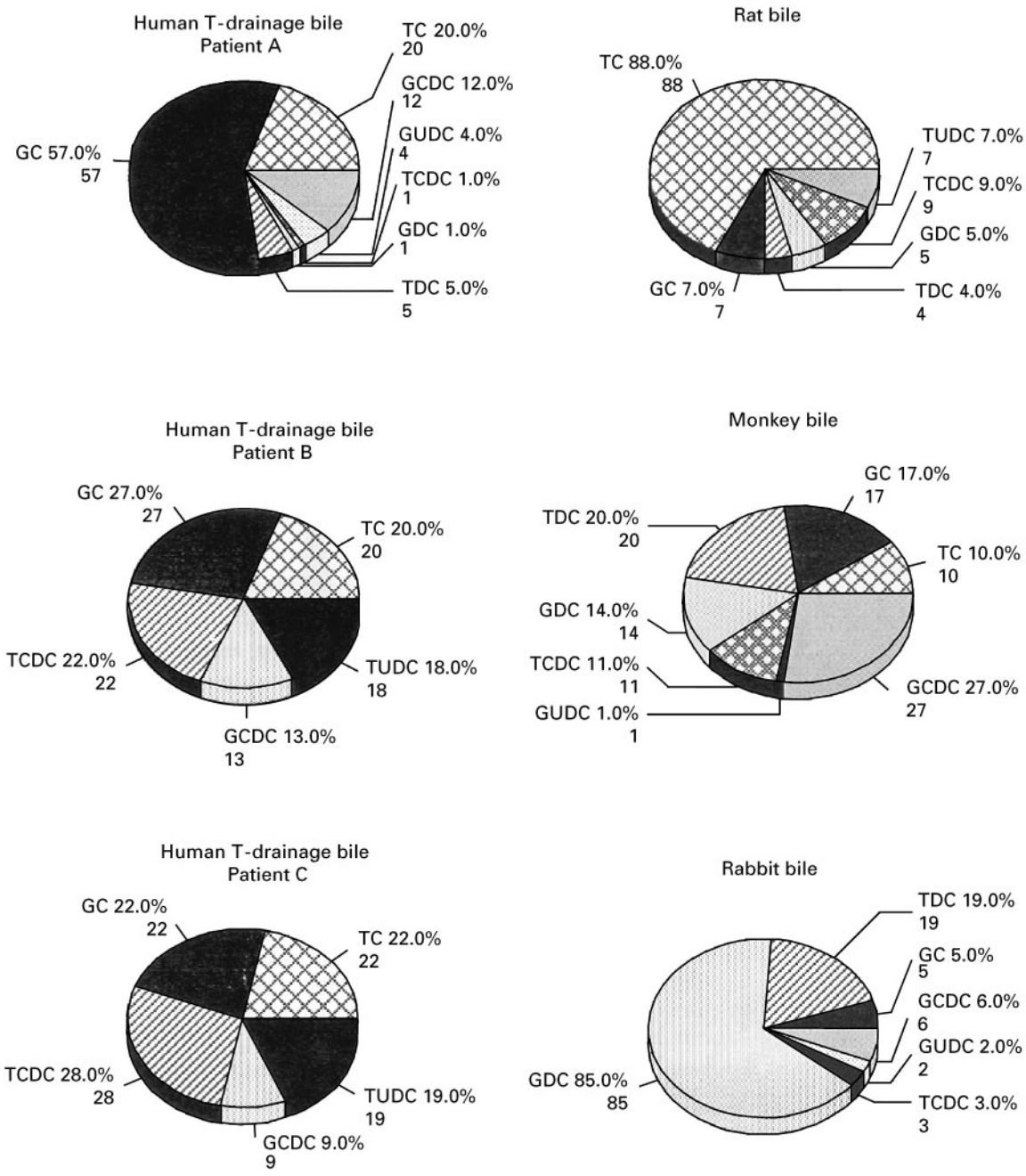

Figure 3 Bile acid patterns obtained from three samples of human T-drainage bile and from the bile of other animals. Key as for Figure 1, plus GDC, glycodeoxycholic acid and TDC, taurodeoxycholic acid. (From Müller *et al*. 1992.)

mixture for the separation of conjugated bile acids on reversed-phase TLC plates is provided. TLC conditions are similar to an HPLC method described for the separation of conjugated bile acids, and may be helpful in comparison of sample analysis by different analytical techniques or in cases where simultaneous separation of bile acids and drug metabolites is required.

In all the separation systems described temperature plays an important role in resolution.

Although room temperature (20–22°C) allows satisfactory separation employing the systems described, in some cases decreasing the temperature improves separation and band shape. Depending on the sample concentration and composition the influence of temperature on separation resolution should be taken

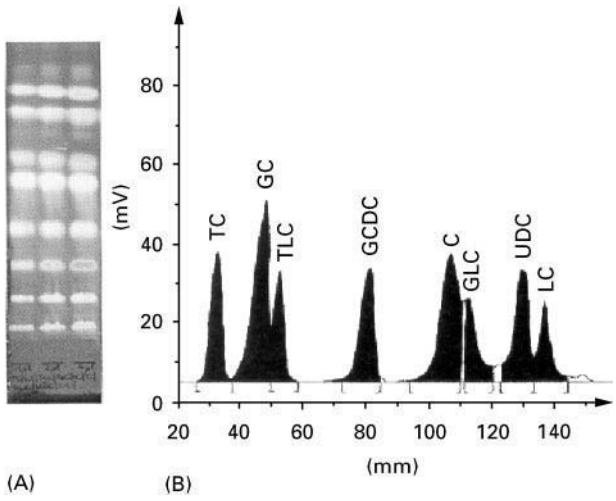

Figure 4 (A) Thin-layer chromatogram of increasing amounts (1, 2 and 4 μL of 4 mg mL^{-1} solutions) of bile acid standards TC, GC, TLC, GCDC, C, GLC, UDC and LC. (B) Fluorescence scan of the left lane of the chromatogram. Key as for Figure 1. (From Müller *et al*. 1992.)

into account as a critical factor for optimization of the chosen solvent system.

Numerous detection methods for bile acid visualization on TLC plates after separation have been described and two principally different systems are in use. Detection methods using reagents which form coloured complexes have the advantage that bands can be visualized directly. A universal detection reagent for a vast amount of biological substances is anise aldehyde dissolved in sulfuric acid. Spraying or dipping of the developed TLC plates followed by incubation at 125°C generates spots of different colour which are stable for some time. However, the low specificity of this reagent may cause problems in the analysis of samples with a high content of other components or analytes. Spots can also be visualized under ultraviolet (UV) light, which increases sensitivity. Individual bile acids can be identified by their different colours (**Figure 5**).

Molybdatophosphoric acid is another reagent often used for detection of biological components and forms blue bands on a yellow ground. Since colour intensity of the visualized spots is not stable over a long period of time, quantification must follow immediately after development of the colour.

The sensitivity of detection of bile acids on TLC plates can be increased dramatically by using a reagent system which forms fluorescent bands. $HClO_4$ has been described as giving reproducible fluorescent spots with bile acids on TLC plates, provided a 5% solution of this derivatizing agent is used and UV light of 365 nm excitation wavelength is employed after spraying and appropriate treatment at higher temperature.

According to our experience a reagent mixture of manganese dichloride and sulfuric acid is preferred for detection, owing to the reproducible results and its convenience in use. Plates are dipped in the reagent

Table 1 Solvent systems for bile acid separation

System 1		
Unconjugated bile acids	Isooctane Diethyl ether n-Butanol Acetic acid Mixture 10 + 2.5 + 1 + 1 (v/v)	Room temperature
System 2		
Glycine- and taurine-conjugated bile acids	Chloroform 2-Propanol 2-Butanol Acetic acid Double-distilled water Mixture 30 + 20 + 10 + 2 + 1 (v/v)	Room temperature 2 × 15 cm followed by 4 × 7 cm
System 3		
All bile acids	Chloroform Methanol Acetic acid Mixture 85 + 20 + 9 (v/v)	Room temperature Chamber saturation
System 4		
Glycine- and taurine-conjugated bile acids	Acetonitrile (90%) 0.01 M Ammonium carbamate pH 7.3 Mixture 55 + 45 (v/v)	Room temperature Separation on RP8 plates

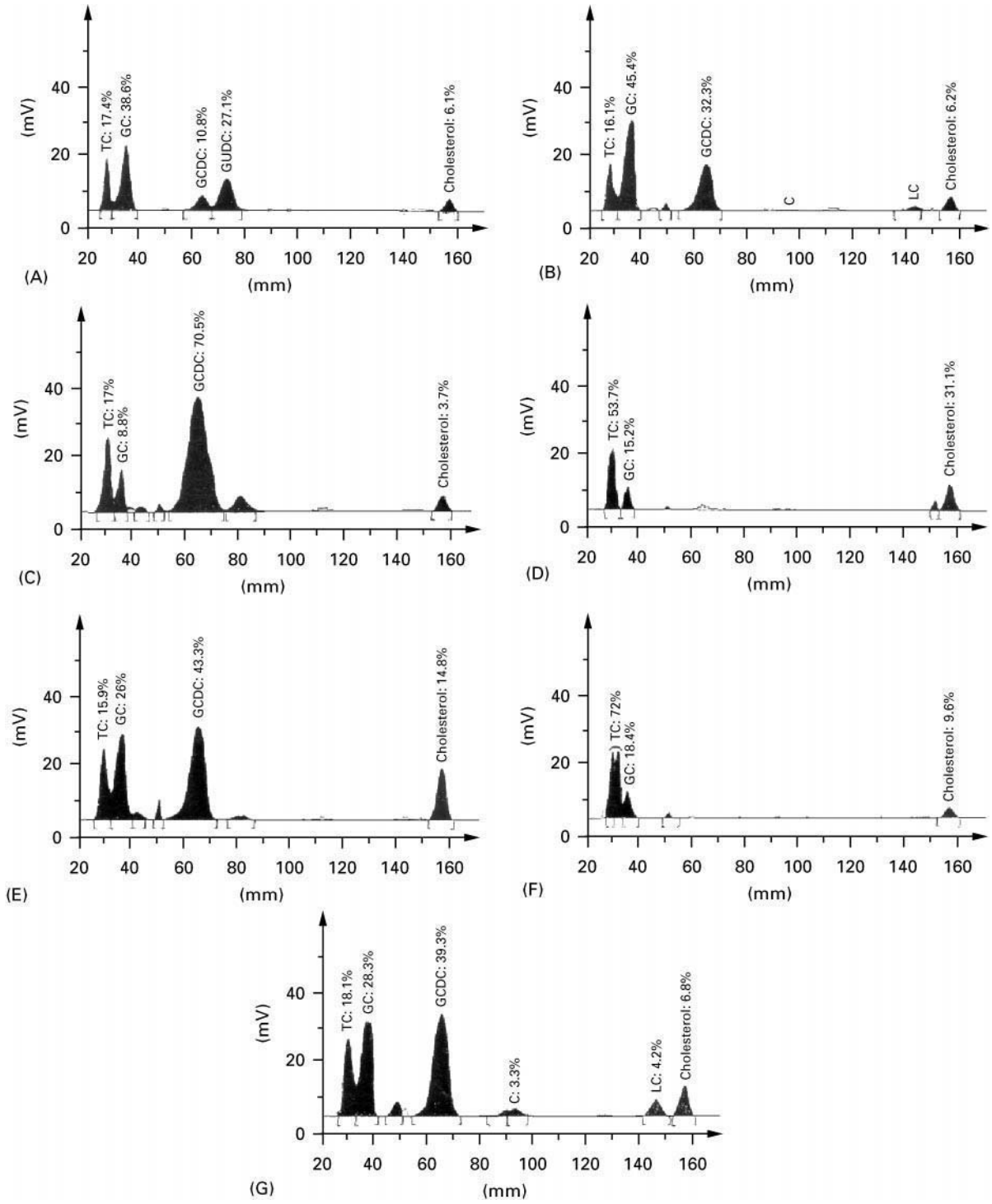

Figure 5 Fluorescence scans of bile specimens from Figure 1: (A) human bile II; (B) human bile I; (C) rabbit bile; (D) pike bile; (E) monkey bile; (F) carp bile; (G) ox bile. Key as for Figure 1. (From Müller *et al*. 1992.)

and heated to 110°C for 10–15 min. Bile acids react with the reagent with the formation of yellow or brown coloured spots. Under UV light (365 nm) blue or yellow fluorescing bands can be detected. Bile acids can be differentiated by their respective colour. The detection sensitivity for the bile acids under UV conditions is 2–5 ng and is therefore about 1000-fold higher compared to visible light (**Figure 4**).

Cholesterol

Cholesterol and cholesterol esters are important bile components. Several solvent systems for the

Table 2 Solvent systems for separation of cholesterol and cholesterol esters on silica gel plates

System 1	Room temperature
Chloroform	Chamber saturation
Methanol	
Acetic acid	
Mixture 85 + 20 + 9 (v/v)	
System 2	Room temperature
Chloroform	
Acetone	
Mixture 85 + 15 (v/v)	
System 3	Room temperature
Cyclohexane	
Diethyl ether	
Mixture 50 + 50 (v/v)	
System 4	Multiple plate development
Methanol	
Diethyl ether	
n-Hexane	

separation of these steroids exist, but only some of them are suitable for the analysis of cholesterol in complex biological fluids. In **Table 2** four commonly used methods are shown. The mixture of chloroform and acetone is described for the detection of cholesterol in serum samples and can successfully be adapted to bile analysis. In system 4 three organic solvents with decreasing polarity are suggested for three consecutive development steps and can be used in automated multiple development devices.

If simple and fast one-step analysis of cholesterol and bile acids is required, solvent system 1 combined with the above described manganese detection reagent should be used, since cholesterol can be separated and identified easily by fast migration and its orange fluorescence. Other staining reagents containing either trichloracetic acid or 8-anilinonaphthalinesulfonic acid ammonium salt have been shown to react with cholesterol to fluorescent bands, and numerous other universal detection systems are described in the literature. Adaptation to bile analysis seems to be possible in most cases.

Use of the NCS reagent (1,2-naphthochinone-2-sulfonic acid sodium salt) leads to purple bands which can be easily detected and quantified with detection limit at 5 ng cholesterol.

Phospholipids

As for the other bile components, several solvent systems for separation of phospholipids are known. However, most of them were not developed for the analysis of bile samples, but for other biological materials or mixtures of purified standard compounds. Due to the fact that the most abundant phospholipid in bile samples is lecithin, three solvent systems able to separate phospatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylglycerol are shown in **Table 3**.

Some of the most commonly used and versatile detection reagents described in the sections above are also suitable for phospholipid detection both under UV and visible light. Detection with the 2,7-difluorescein reagent increases sensitivity and allows reliable and satisfactory quantification. Detection by a reagent mixture containing ammonium molybdate, sulfuric acid and ascorbic acid results in blue bands which can be easily detected and quantified by a TLC scanning device at a wavelength of 620 nm.

Conclusion

For decades, TLC has proven its advantages as a fast, inexpensive, reliable as well as highly reproducible technology in the analysis of bile, bile acids and bile acid derivatives. Especially in bile acid and cholesterol analysis several separation and detection systems have been developed which have shown convincing sensitivity and overall resolution.

Solvent systems allowing the simultaneous detection of bile acids, cholesterol and drug metabolites in a one-step analysis give a significant time advantage at reduced cost. In addition, the separation system and overall conditions can easily be adapted to the respective analytical problem in most cases.

Although the quantitative detection of phospholipids in bile does not play a key role in bile

Table 3 Solvent systems for phospholipid separation on silica gel plates

System 1	
Chloroform	Room temperature
Methanol	
Ammonia (conc.)	
Mixture 60 + 35 + 3 (v/v)	
System 2	
Petroleum ether	Room temperature
Chloroform	
Methanol	
Acetic acid	
Mixture 30 + 50 + 15 + 10 (v/v)	
System 3	
Cyclohexane	Room temperature
Isopropanol	
Double-distilled water	
Mixture 30 + 40 + 6 (v/v)	

analysis at the moment, there is still a need to develop accurate TLC methods for detailed separation of these lipids. Further investigations on the use of new TLC materials in the quantitative analysis of bile components are needed, as well as the adaptation of TLC methods to automated devices. Nevertheless, compared to other analytical tools TLC is the method of choice for fast routine use.

See also: **II/Chromatography: Thin-Layer (Planar):** Densitometry; Layers; Mass Spectrometry; Spray Reagents. **III/Bile Acids:** Liquid Chromatography; Gas Chromatography. **Clinical Diagnosis: Chromatography. Lipids:** Liquid Chromatography; Gas Chromatography; Thin-Layer (Planar) Chromatrography. **Neonatal Metabolic Disorders: Detection: Thin-Layer (Planar) Chromatography**.

Further Reading

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BIOANALYTICAL APPLICATIONS: SOLID-PHASE EXTRACTION

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Introduction

The quantification of drugs and metabolites in biological fluids (e.g. plasma, serum and urine) is one of the most active research fields in clinical and pharmaceutical analysis. Drug bioanalysis is important in clinical chemistry to demonstrate optimal drug therapy, because plasma drug concentrations relate to the therapeutic or toxic effects of a drug. Knowledge of the plasma drug concentration is used to determine why a patient does not respond to drug therapy or why a drug causes an adverse effect. Dosage adjustment by the physician is then warranted. Drug bioanalysis is important in pharmaceutical research to determine the pharmacokinetics and metabolic biotransformation of new drug molecules. It is a technique used throughout the development of all new drugs. In particular, during drug discovery, bioanalysis yields essential data that is used in the decision-making process of whether or not a new molecule should be a candidate for further development.

This chapter discusses the utility of solid-phase extraction (SPE) in comparison with other drug sample preparation techniques for bioanalysis. Several applications of SPE will be summarized in the clinical setting for therapeutic drug monitoring, and in the pharmaceutical research setting for drug discovery and development. The separation and detection techniques used for bioanalysis will be examined, contrasting the use of high-pressure liquid chromatography (HPLC) in the clinical setting and the rapid proliferation of HPLC combined with tandem mass spectrometry (liquid chromatography/mass spectrometry/mass spectrometry or LC/MS/MS) for specific and sensitive detection of drugs for pharmaceutical bioanalysis.

Drug Sample Preparation Techniques

A reliable analytical method is achieved with efficient sample preparation, adequate chromatographic separation, and a sensitive detection technique. Detailed and exact guidelines exist for the validation of bioanalytical methods, which meet requirements agreed to by the Food and Drug Administration. The sample preparation step is an important component of each overall bioanalytical method, as it

- often concentrates an analyte to improve its limits of detection,
- removes unwanted matrix components that can cause interferences upon analysis, thus improving method specificity, and
- frees the analyte from matrix components so that it can be placed into a solvent suitable for injection into the chromatographic system.

Liquid/liquid extraction

A common sample preparation procedure used to isolate drug analytes from biological matrices is liquid/liquid extraction (LLE). It is quite effective at removing salts and proteins; water-soluble endogenous components often remain in the aqueous phase.